Determination of Adenosine 3':5'-Cyclic Monophosphate in Cerebral Tissues by Saturation Analysis

ASSESSMENT OF A METHOD USING A BINDING PROTEIN FROM OX MUSCLE

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1. The Gilman (1970) procedure for determining cyclic AMP (adenosine 3':5'-cyclic monophosphate) by saturation analysis gave erroneous results when applied to the analysis of extracts of whole brain or preparations of membrane fragments from brain.
2. The extracts contained a non-diffusible factor, which enhanced the binding of cyclic AMP by the muscle protein fraction.
3. Extracts also contained material which inhibited binding, but net inhibition of binding was only observed when relatively concentrated extracts were analysed.
4. The error introduced by the factors modifying binding could be eliminated by incorporation of unlabelled internal standards in the unknowns. The design adopted enables a statistical estimate to be made of the standard error of a single assay.
5. The modified assay was used to determine bound cyclic AMP and adenylate cyclase activity in cerebral membrane fragments. Five preparations of synaptic membrane fragments contained less than 3.5 pmol of cyclic AMP/mg of protein; a microsomal fraction from rat contained 65 pmol of cyclic AMP/mg of protein.

Several elegant methods for determining cyclic AMP (adenosine 3':5'-cyclic monophosphate) by saturation analysis are now available. In general these methods depend on the capacity of protein fractions from skeletal muscle (Gilman, 1970) or adrenal gland (Walton & Garren, 1970; Brown *et al.*, 1971) to bind the cyclic nucleotide; they possess the advantage of combining simplicity with sensitivity, but are susceptible to error if the tissue extract contains substances that interfere with the binding reaction.

A striking example of such interference was encountered by us in applying the method of Gilman (1970) to the analysis of extracts of washed membrane fragments from brain for their content of bound cyclic AMP. These extracts, which were necessarily more concentrated than usual because the quantity of cyclic AMP bound to membrane fragments is very low, were found to contain a substance which inhibited the binding reaction. The inhibition was not due to ATP or other soluble cell constituents, since the membrane preparations had been thoroughly washed in the centrifuge with hypo-osmotic solutions before extraction. Further, in devising a procedure to correct for this interference it was found that more dilute extracts of cerebral tissue contained a non-diffusible substance which enhanced the binding of cyclic AMP by the muscle protein. To enable extracts of widely different concentrations to be analysed without preliminary purification, the Gilman (1970) procedure was redesigned as described in this paper.

Experimental Methods

Materials

The ammonium salt of [8-³H]adenosine 3':5'-cyclic monophosphate (specific radioactivity 2×10^3 – 3×10^3 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Cellulose acetate filter discs were purchased from the Oxoid Division of Oxo Ltd., London S.E.1, U.K. Reagent-grade (NH₄)₂SO₄ used in the preparation of the binding protein was recrystallized once from EDTA solution made slightly alkaline with NH₃. Shin muscle from ox was obtained from a slaughter-house 45 min after death of the animal and brought to the laboratory packed in ice.

Preparation and properties of binding protein

This was prepared from the muscle by the simplified procedure of Gilman (1970), except that the final extract was fractionated on a column (30 cm $\times$ 2.6 cm) of Whatman DE52 cellulose instead of Whatman DE11 cellulose. Separation of the two main peaks of binding activity was excellent. The yield of protein from the last peak was 0.04–0.1 mg/g of muscle (three preparations).

The preparations bound 0.15–0.18 pmol of cyclic AMP/ μ g of protein when assayed with concentrations of nucleotide saturating the binding sites, under

the standard conditions indicated below. This compares with a value of $0.3 \text{ pmol}/\mu\text{g}$ of protein quoted by Gilman (1970) for his preparation. The affinity or binding capacity of our preparations were not increased by the inclusion of 1 mM -cysteine, 1 mM - β -mercaptoethanol or 50 mM -EDTA in the reaction mixture. As a routine we have used $4.5 \mu\text{g}$ of binding protein in a final volume of 0.2 ml , and the relation between amounts of cyclic $[^3\text{H}]\text{AMP}$ added and bound under these conditions is shown in Fig. 1. With 4 pmol of cyclic AMP present the binding reaction was complete in 30 min at 0°C and the amount of radioactivity bound was then stable for a further 90 min . Dilution of the reaction mixture to 1 ml after 60 min with 20 mM -potassium phosphate buffer, $\text{pH} 6.0$, resulted in a loss of about 20% of the bound label within 0.5 min , but the remainder was stable for several hours at 0°C . These characteristics of the binding reaction are essentially the same as those reported by Gilman (1970). We were unable, however, to confirm his observations that the inclusion in the reaction mixture of a heat-stable inhibitor of protein kinase activity prepared from ox muscle increases the affinity of the binding protein for cyclic AMP. Three different inhibitor preparations, made from ox shin muscle exactly as described by Gilman (1970), decreased affinity markedly, although they did (as reported by Gilman, 1970) prevent the loss of label that occurred on dilution. With two preparations a small increase in the total number of binding sites available was observed. In view of this we have not followed the recommendation of Gilman (1970) to include the kinase inhibitor preparation in the assay system.

Preparation of membrane fragments

Microsomal preparations from ox cerebral cortex were made by the procedure of Rodnight (1970) and synaptic membrane fragments from the same source by the procedure of Rodnight *et al.* (1969).

Extraction of tissue samples

Two methods were used.

(a) In most experiments samples were extracted with 0.1 M -HCl at 100°C for 2 min as described by Butcher *et al.* (1965). Suspensions of membrane fragments were treated at a protein concentration of $5\text{--}10 \text{ mg/ml}$; fresh tissue was homogenized at a concentration of 100 mg of tissue/ ml of acid. After cooling and centrifuging, the extracts were adjusted to $\text{pH} 4.0$ by addition of 1 M -sodium acetate-acetic acid buffer, $\text{pH} 4.6$, care being taken to ensure that the final concentration of acetate in the binding assay was less than 100 mM , since higher concentrations were found to inhibit the binding reaction.

In our hands the above procedure was superior to

that of Kakiuchi *et al.* (1969), in which the tissue is extracted with cold HCl and centrifuged without heating to 100°C . This was found to give lower values for the cyclic AMP content of both whole brain tissue and membrane preparations, and also lower recoveries of added cyclic AMP than did the procedure using hot HCl. Both procedures extracted similar amounts of material interfering with the binding reaction.

No results using cold HCl for extraction are reported in the present paper.

(b) In some experiments samples were extracted with 10% (w/v) trichloroacetic acid. After centrifuging, the supernatants were extracted with $4 \times 2 \text{ vol.}$ of water-saturated diethyl ether and traces of ether were removed by warming.

Some of the crude HCl extracts were purified by a procedure based on that of Kakiuchi & Rall (1968). The extract, adjusted to an HCl concentration of 25 mM and a vol. of 20 ml , was pumped through a

Fig. 1. Relation between cyclic $[^3\text{H}]\text{AMP}$ added and label bound in the presence and absence of tissue extract

The reaction mixture (0.2 ml) contained 50 mM -sodium acetate-acetic acid buffer ($\text{pH} 4.0$), $4.5 \mu\text{g}$ of binding protein and cyclic $[^3\text{H}]\text{AMP}$ as indicated. Label bound to protein was determined by the procedure given in the text. o, Cyclic AMP bound in the absence of added tissue extract; Δ , cyclic AMP bound in the presence of a dialysed (see text) hot HCl extract derived from $90 \mu\text{g}$ of tissue protein (whole cerebral cortex of frozen ox brain). Each point is the mean of three observations.

column (6cm×0.9cm) of Dowex 50 (X8; H⁺ form; 100–200 mesh) equilibrated with 25mM-HCl. The column was then washed with 25ml of 25mM-HCl, which was discarded, and a further 60ml of 25mM-HCl, which was collected and freeze-dried. The residue was dissolved in a small volume of water. Recovery of added cyclic AMP was 100±10%, provided that chromatography columns treated with silicone (Siliclad; Clay Adams Inc., New York, N.Y. 10010, U.S.A.) were used; without this precaution much lower recoveries were obtained, presumably owing to the adsorption of the nucleotide on glass.

Standard assay procedure

The procedure adopted was a modification of that of Gilman (1970) using hot HCl extracts. The binding reaction was carried out in plastic test tubes of capacity 1 ml. To each tube the components of the reaction mixture were added in the following order: 40μl of 250mM-sodium acetate buffer, pH4.0; 50μl of 80nM-cyclic [³H]AMP (2250 d.p.m./pmol); an appropriate vol. (see below) of standard unlabelled cyclic AMP; 50μl of sample; enough water to make the final volume after addition of binding protein 0.2ml; 10μl of binding protein (0.45mg/ml), added from a micro-syringe fitted with an accurate dispensing device. Before and after addition of the binding protein, the tubes were briefly agitated to ensure thorough mixing of the contents. The tubes were placed in an ice-water mixture for 60min or longer and the contents were then diluted to 1 ml with 20mM-KH₂PO₄ buffer (adjusted to pH6.0 with KOH). After at least 1 min, the contents were filtered through Oxoid cellulose acetate filter discs (grade 0.45 UP, 2.5cm diam.) held in standard Millipore filtration equipment. The tubes and filter discs were washed with 8ml of 20mM-KH₂PO₄ buffer, pH6, in several portions. A period of about 1h was required to filter 36 samples. The discs were then placed in counting vials, dried in an oven at 60–70°C for 10min and cooled, and 10ml of the scintillation fluid used by Weller & Rodnight (1971) was then added. After it had been checked that the discs remained situated in the well of the vial, their radioactivities were counted in a Packard spectrometer at an efficiency of 20%. Blanks conducted in the absence of binding protein gave no detectable binding of radioactivity to the filters.

Unlike the Millipore filter discs used by Gilman (1970), the Oxoid filters are insoluble in methyl- or ethyl-Cellosolve. However, the efficiency achieved with the method adopted was acceptable. Application of the channels-ratio method showed no difference in counting efficiency between vials, and the efficiency was independent of minor variations in the position of the filter discs. No radioactivity was lost

to the scintillation fluid, which could therefore be used repeatedly after routine checks for accidental contamination.

Results and Discussion

Design of assay

Unlabelled internal standards, added to the unknown samples, are an essential feature of the present design. To obtain an acceptable degree of precision and an estimate of the error in the assay, it was generally necessary to analyse three equal samples of the unknown in triplicate, making a total of 0.45ml of extract required. Standards (2 and 4pmol) of unlabelled cyclic AMP were incorporated in the reaction mixture for two of the triplicate samples. We refer to this design below as the standard 3-point assay. For many routine purposes, however, a 2-point assay, with duplicate samples with and without 4pmol of unlabelled standard added to the unknown, was satisfactory. Both assay designs gave the best results in the range 2–10pmol of cyclic AMP.

External standards were only analysed when constructing the calibration graph (Fig. 2) with fresh preparations of binding protein and for occasional checks during the course of its period of use.

Calculation of results

For the 3-point assay the following graphical method was used. The reciprocals of the radioactivity bound (d.p.m.) were calculated and the means for each point plotted against the amount of standard cyclic AMP added (pmol). This gave a linear plot which, with the pure (external) standards, or with tissue extracts containing no cyclic AMP, cut the abscissa in the negative direction at a point equal to 4pmol of cyclic AMP, i.e. the molar quantity of cyclic [³H]AMP present. Similar plots for extracts containing cyclic AMP cut the abscissa at 4 + the number of pmol of cyclic AMP in the volume of extract analysed. Typical plots are illustrated in Figs. 2, 3 and 5.

The graphical method was derived from the following considerations: If [A] = pmol of cyclic [³H]AMP present, [S] = pmol of unlabelled cyclic AMP added as external standard for constructing the calibration graph, [iS] = pmol of unlabelled cyclic AMP added as internal standard to the unknowns, [U] = pmol of cyclic AMP present in unknown, [B] = pmol of cyclic AMP bound, $X = \text{d.p.m. of total radioactivity present}$ and $Y = \text{d.p.m. of radioactivity bound to the protein}$, then, as enough cyclic AMP to saturate the protein is always present, $Y = [B]X/([A] + [S])$ for the external-standard plot and $Y = [B]X/([A] + [U] + [iS])$ for the unknown plot

Fig. 2. Typical standard plot of unlabelled cyclic AMP added against the reciprocal of the radioactivity bound (d.p.m.)

The conditions given in the text were used. Each point is the mean of three observations. The line drawn through the points is the best fit calculated as described in the text.

Fig. 3. Determination of cyclic AMP in a membrane preparation

Standard conditions given in the text were used. ○, Standard plot (without addition); △, plot in the presence of a hot HCl extract of synaptosome membrane fragments derived from 500 μg of tissue protein; □, plot in the presence of extract with the addition of 1.5 pmol of extra cyclic AMP to each tube. This extract contained no endogenous cyclic AMP. Note that the lines drawn through the symbols △ and □ are parallel and that the latter cuts the abscissa at -5.5, i.e. at a point equal to $-([A] + [U])$ since in this case $[U] = 1.5$ pmol. Each point is the mean of three observations.

derived from unknowns + internal standards. Hence for the external standards $1/Y = ([A]/[B]X) + ([S]/[B]X)$, and by plotting $1/Y$ against $[S]$ a straight line is obtained of slope $1/[B]X$, crossing the abscissa at a value equivalent to $-[A]$. Similarly for unknown

samples, plotting $1/Y$ against $[iS]$ gives a straight line crossing the abscissa at $-([A] + [U])$. Confirming theoretical expectation, it was found that if insufficient cyclic AMP was present to saturate the protein the plots were non-linear.

For results derived from pure standards the treatment described above has been experimentally verified many times with three different preparations of binding protein. For example, in the experiment shown in Fig. 2, the calculated best fit for the regression line (see below) cut the abscissa in the negative direction at 4.09 pmol of cyclic AMP (the small deviation from the theoretical value can be accounted for by the error involved in pipetting the labelled cyclic AMP). When tissue extracts were analysed, however, linear plots were still obtained but with a different slope to that observed with pure standards. This was due to the occurrence in the extracts of material which altered the capacity of the protein to bind cyclic AMP. Recovery experiments with extracts containing no endogenous cyclic AMP showed that the change in slope did not invalidate the assay, as the experimental plots still cut the abscissa at the theoretical value of $-(A+U)$. The 3-point assay design therefore enables crude extracts to be analysed, while at the same time giving an indication of the extent to which the extracts contain interfering material.

Calculation of results for the 2-point design can be made either graphically as described above or from the equation:

$$[U] = \{C([A] + [iS]) - [A]\} / (1 - C)$$

where C is the ratio of radioactivity bound with the unknown sample containing internal standard (m) to the radioactivity bound with the unknown sample alone (n). {This follows since $m = [B]X / ([A] + [iS] + [U])$ and $n = [B]X / ([A] + [U])$; therefore $C = ([A] + [U]) / ([A] + [iS] + [U])$, which when solved for $[U]$ gives the above expression.}

It can be shown on theoretical grounds that these procedures for calculating the results are only valid in the absence of substances competing with cyclic AMP for binding sites on the protein. In the presence of a competitive inhibitor a non-linear reciprocal plot is obtained, as is shown below for a mixture of cyclic AMP and cyclic GMP (see Fig. 7). (However, in the very improbable case of the binding protein having the same affinity for the competitor as for cyclic AMP a linear response is expected and the assay would determine the competitor as cyclic AMP.)

Statistical treatment of results

The 3-point assay gives sufficient results to permit a statistical evaluation to be made of the error in a single determination. As indicated above the plot for the unknown sample is derived from the equation $1/Y = [iS]/[B]X + ([A] + [U])/[B]X$. Standard regression analysis then gives the s.d. of $[U]$ as $a/b\sqrt{\{(\text{s.d. of } a)^2/a^2\} + \{(\text{s.d. of } b)^2/b^2\}}$, where a = the intercept on the ordinate and b = the slope of the regression line.

It may be noted in passing that b is given by the expression:

$$\frac{N\left(\sum \frac{[iS]}{Y}\right) - \left(\sum [iS]\right)\left(\sum \frac{1}{Y}\right)}{N\left(\sum [iS]^2\right) - \left(\sum [iS]\right)^2}$$

and its s.d. by:

$$\sqrt{\left\{\frac{1}{(N-2)}\left\{\frac{N\sum\left(\frac{1}{Y}\right)^2 - \left(\sum \frac{1}{Y}\right)^2}{N\left(\sum [iS]^2\right) - \left(\sum [iS]\right)^2} - b^2\right\}\right\}}$$

where N = the total number of observations. The intercept $a = \{\sum(1/Y) - b\sum[iS]\}/N$ and its s.d. = s.d. of $b\sqrt{\sum[iS]^2/N}$.

Investigation of interfering material

We first became aware of this interfering material when analysing extracts of synaptosomes for bound cyclic AMP. From determinations by Gilman's (1970) procedure, it appeared that the extracts contained about 15 pmol of cyclic AMP/mg of protein. However, no cyclic AMP could be detected in the extracts after purification by the procedure of Kakiuchi & Rall (1968). Analysis of the crude extracts by the present procedure by using internal standards confirmed this result and drew our attention to the material in the extracts which interferes with binding. In this case the extract was found to inhibit binding, but in subsequent work, when smaller amounts of extract were analysed, the opposite effect was found. Fig. 4 shows that net inhibition of binding by an extract of membrane fragments occurred when a quantity of extract equivalent to more than 600 μ g of membrane protein was taken for analysis; lower quantities of extract enhanced binding, the maximum effect occurring when an amount of extract derived from 80 μ g of protein was used. Therefore in the former case the slope of the regression line for the unknown was greater than for the standard, whereas in the latter case it was less (Figs. 3 and 5). However, since the linearity of the plots was independent of the amount of extract analysed, the procedure was applicable to tissue samples of widely different cyclic AMP contents. In most applications the slope of the unknown regression line will presumably be decreased, since it is only rarely that cyclic AMP contents of less than 0.5 nmol/g of tissue are likely to be encountered.

The results illustrated in Figs 3, 4 and 5 were obtained with the preparation of binding protein used for most of this work. A second preparation, which was only one-half as active and therefore required the addition of 9 μ g of protein sample to yield sufficient bound radioactivity, gave a rather different result. With this second preparation, the slope of the regression line increased much less sharply with

increasing sample concentration than it did with the more active preparation. Thus, with up to an equivalent of 1mg of protein in the sample the slope was less than that obtained with the calibration graph of pure standards, indicating that enhancement instead of inhibition of binding was occurring; at an equivalent of 2mg of protein/sample, however, the two lines were parallel, suggesting that at this concentration of extract the inhibitory factor just balanced the enhancing factor. With the first preparation this balancing of the two factors occurred at an equivalent of about 600 μ g of protein, as shown in Fig. 4. In view of the rather different behaviour of this less-active preparation it was gratifying to find that the two preparations gave, within experimental error, the same results for the cyclic AMP content of extracts of membrane fragments.

The interfering material occurred equally in extracts of whole brain or of membrane fragments, and in extracts made with HCl at 100°C or 20°C or with trichloroacetic acid. The material responsible for the stimulating effect found at low concentrations of an HCl extract was not removed by dialysis for 18 h against 100vol. of water with four changes (Fig. 1), nor was it retained by cellulose ester filters (0.45 μ m pore size). We have not determined whether the inhibitory effect is similarly removed by dialysis. All the interfering material could, however, be removed by treatment with Dowex 50 (H⁺ form), making it feasible, if desired, to purify extracts by the procedure

of Kakiuchi & Rall (1968). We do not recommend purification as a routine since it considerably increases the time required for the assay, even when the stan-

Fig. 4. Effect of different amounts of tissue extract on the binding of label

Binding was measured under standard conditions in the presence of various amounts of hot HCl extract of synaptosome-membrane fragments. Inhibition of binding by this extract was less than observed with the extracts used in Figs. 3 and 5, but more than that found with a less-active preparation of binding protein.

Fig. 5. Removal by Dowex 50 resin of material inhibiting binding

Extracts of synaptosome membrane fragments were made with hot HCl and purified by passage through a column of Dowex 50 as described in the text. ○, Standard plot without addition; ■, plot in the presence of a quantity of original extract before purification equivalent to 300 μ g of tissue protein; □, plot in the presence of purified extract (equivalent to 375 μ g of tissue protein) with addition of 5 pmol of cyclic AMP to each tube; ▲, plot in the presence of a quantity of the original extract equivalent to 20 μ g of tissue protein and illustrating enhancement of binding. Note that the line for the purified extract is nearly parallel to the standard plot and cuts the abscissa at -9, i.e. at a point equal to $-(A+U)$, since in this case $[U] = 5$ pmol. Each point is the mean of three observations.

standard Gilman (1970) procedure is used for the final determination of cyclic AMP in the purified extract. The properties of the inhibitory material suggest that it probably consists of an acid-soluble peptide (or peptides), since other agents known to interfere with binding, such as nucleotides (Gilman, 1970), would have been largely removed by dialysis.

Effect of ATP and cyclic GMP on the assay

Gilman (1970) reported a 50% inhibition of binding by 1 mM-ATP, but no inhibition by 0.1 mM-ATP. We have confirmed this finding. Unfortunately the internal-standard design does not adequately correct for inhibition of binding from this source. For example, inhibition of the binding of pure standards of cyclic AMP by 0.5 mM-ATP was apparently non-competitive, since a straight line was obtained; however, although the regression line was linear the

intercept on the abscissa occurred at -2.8 ± 0.3 , a value which differed significantly ($P < 0.001$) from the theoretical value of -4 (Fig. 6). At 0.2 mM-ATP the error was less serious, but still statistically significant: the calculated regression line cut the abscissa at -3.6 ± 0.3 ($P = 0.02$ for difference from -4). In several experiments a high concentration of ATP (1 mM) gave a non-linear response, but, as the means always had large s.d. values, it was not possible to determine with any confidence the shape of the line. In practice interference by ATP is not serious, since, as pointed out by Gilman (1970), the sensitivity of the assay is sufficient to permit the dilution of tissue extracts to a degree sufficient to give an ATP concentration well below 0.1 mM.

Cyclic GMP may be expected to inhibit the binding of cyclic AMP competitively. Confirming theoretical expectation, the addition of $5 \mu\text{M}$ -cyclic GMP to the assay gave a non-linear response, inhibition of

Fig. 6. *Effect of ATP on the slope*

The slope was determined under standard conditions in the absence (○) or presence (Δ) of 0.5 mM-ATP. Each point is the mean of six observations, with vertical bars indicating the s.d. In both cases the lines drawn through the points are the best fits calculated as described in the text.

Fig. 7. Effect of cyclic GMP on the assay

A standard plot was constructed in the absence (○) or presence (Δ) of $5 \mu\text{M}$ -cyclic GMP. Each point is the mean of six observations with vertical bars indicating the S.D.

binding decreasing with increasing concentration of cyclic AMP (Fig. 7).

Error of the assay

The standard error of the 3-point assay was derived by dividing the S.D., calculated as indicated above, by $\sqrt{(9)}$, i.e. the number of individual observations made in the assay. In 17 such assays made on hot HCl extracts of fresh cerebral tissue, cortical slices after incubation, and membrane fragments, the standard errors so obtained, expressed as percentages of the mean cyclic AMP contents, ranged from $\pm 0.5\%$ to $\pm 19\%$, with a mean of $\pm 4.9\%$. The range of cyclic AMP contents in this series was 2.68–292 pmol/mg of protein. This value of $\pm 5\%$ is probably an overestimate of the percentage error, since the series includes results obtained by three workers and also a number of assays in which the cyclic AMP content of the extract was outside the optimum range of 2–10 pmol/sample analysed.

With the 2-point assay five determinations in parallel of two dilutions of an extract of fresh cerebral cortex gave the following mean values, in pmol ($\pm$ S.D.)/50 μl of dilution: extract dilution A, 1.16 ± 0.38 (range 0.7–1.6); extract dilution B, 9.7 ± 1.48 (range 7.7–12.7). From these values the standard error of the 2-point assay appears to be in the range $\pm 7\%$ to $\pm 15\%$.

Investigation showed that about one-half of the

error was incurred in setting up the assay, which is understandable since this stage involves pipetting accurately microlitre quantities of four different solutions. The error involved in the filtering stage was of the order of $\pm 2\%$ and in subsequent stages only about $\pm 1\%$.

Cyclic AMP content and adenylate cyclase activity in membrane preparations from brain

Values for several preparations of synaptic membrane fragments and a microsomal fraction obtained by the present procedure are given in Table 1. These compare reasonably with values of 100.9 pmol of cyclic AMP/mg of protein for a microsomal fraction and 8.3 pmol/mg of protein for a 'synaptic ghost' fraction, both from rat brain, reported by Goldberg *et al.* (1970). These authors ascribed the much higher content of cyclic AMP in their microsomal fraction to the presence in the latter of 'trapped' nucleotide, but we are less certain of this explanation of the present results, since the preparation analysed had been extensively washed in the centrifuge with an hypo-osmotic buffer.

Despite their low content of cyclic AMP, synaptic-membrane fragments (stored for 20 days in the frozen state) exhibited adenylate cyclase activity, which was increased in the presence of the phosphodiesterase inhibitor papaverine (Table 2).

Table 1. *Endogenous cyclic AMP content of washed membrane preparations from brain*

Synaptic membrane fragments were prepared by the procedure of Rodnight *et al.* (1969) and a microsomal fraction by that of Rodnight (1970). In both procedures the membrane fragments are washed with 4 mM-imidazole-HCl buffer (pH 7.0) to remove traces of soluble cellular constituents. Cyclic AMP contents were determined by the 3-point assay after extraction of the fragments with hot HCl as described in the text. Results, with the standard errors of the determination as defined in the text, are derived from nine observations.

Membrane preparation	Species	Time stored frozen (days)	Cyclic AMP content (pmol/mg of protein)
Synaptic, 1	Ox	26	<1
Synaptic, 2	Ox	30	<1
Synaptic, 3	Ox	100	3.5 ± 0.2
Synaptic, 4	Ox	5	3.0 ± 0.3
Synaptic, 1	Rat	Unfrozen*	<1
Microsomal, 1	Rat	Unfrozen*	65 ± 5
Microsomal, 1	Rat	28	17 ± 1.3

* Analysed within 48 h of the death of the animal.

Table 2. *Adenylate cyclase activity in synaptic-membrane fragments*

Enzyme activity was determined in a total volume of 1 ml at 20°C. The medium contained 30 mM-tris-HCl buffer (pH 7.4), 1 mM-ATP (magnesium salt) and 1 mg of synaptic-membrane protein (preparation 3, Table 1). The reaction was stopped with 0.1 ml of 1 M-HCl, the mixture heated for 2 min at 100°C and centrifuged. To the supernatant 0.2 ml of 1 M-sodium acetate buffer (pH 4.6) was added. Cyclic AMP was determined by the 3-point assay; results with the standard error of the determination as defined in the text are derived from nine observations.

Time of incubation (min)	Cyclic AMP formed (pmol/mg of protein)	
	No papaverine	+1 mM-Papaverine
0	3.5 ± 0.2	—
5	41 ± 3.3	60 ± 3.3
10	72 ± 1.3	84 ± 6.6
15	60 ± 4.0	150 ± 10.0

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References

- Brown, B. L., Albano, J. D. M., Ekins, R. P. & Sgherzi, A. M. (1971) *Biochem. J.* **121**, 561–562
- Butcher, R. W., Ho, R. J., Meng, H. C. & Sutherland, E. W. (1965) *J. Biol. Chem.* **240**, 4515–4523
- Gilman, A. G. (1970) *Proc. Nat. Acad. Sci. U.S.* **67**, 305–312
- Goldberg, N. D., Just, W. D., O'Dea, R. F., Weir, S. & O'Toole, A. G. (1970) *Advan. Biochem. Psychopharmacol.* **3**, 67–87
- Kakiuchi, S. & Rall, T. W. (1968) *Mol. Pharmacol.* **4**, 367–378
- Kakiuchi, S., Rall, T. W. & McIlwain, H. (1969) *J. Neurochem.* **16**, 485–491
- Rodnight, R. (1970) *Biochem. J.* **120**, 1–13
- Rodnight, R., Weller, M. & Goldfarb, P. S. G. (1969) *J. Neurochem.* **16**, 1591–1597
- Walton, G. M. & Garren, L. G. (1970) *Biochemistry* **9**, 4223–4229
- Weller, M. & Rodnight, R. (1971) *Biochem. J.* **124**, 393–406